GENES FOR CONTROLLING FLORAL DEVELOPMENT IN ORCHID

BACKGROUND OF THE INVENTION

1. Field of the invention

The invention mainly relates to genes for controlling floral development in orchid. In particular, the invention relates to genes for controlling sepal, petal, lip, and stamen developments.

2. Description of the Related Art

Phalaenopsis spp., which has a single axis stem, is a member of *Orchidaceae* family. It is one of the most important ornamental flowers exporting from Taiwan, and has been selected for developing as a delicate agriculture industry. *Phalaenopsis* attracts people by its elegant floral morphology. The *Phalaenopsis* with special floral morphology has a high value in the market.

The flowers of general monocots or eudicots have different types of organs arranged in concentric whorls. The outermost whorl contains sepals and the next whorl contains petals. The third whorl contains the stamens. Furthermore, the female reproductive organs occupy the center of the flower. In the modern molecular biotechnology, a plant having a special floral morphology can be bred by changing the expression of genes for controlling flower development based on the understanding of the regulation mechanism of floral development. In the model plants such as *Arabidopsis* and snapdragon, the genes for controlling floral development and mechanism thereof are well studied (Weigel, D. and Meyerowitz, E. M. 1994. The ABCs of floral homeotic genes. Cell 78, 203-209). In the model plants, the flower controlling mechanism is "ABC model" in which three flower controlling genes A, B and C alone or in combination control the flower organ development. Expression of A alone specifies sepal

formation. The combination of AB specifies the development of petals, and the combination of BC specifies the stamen formation. Expression of C alone determines the development of carpels (Theissen *et al.*, 2000. A short history of MADS-box genes in plants. Plant Mol. Biol. 42, 115-149).

These genes A, B and C are all transcription factor genes and the gene products thereof have a MIKC-type domain structure comprising a MADS-box (M) domain, an intervening (I) domain, a keratin-like (K) domain, and a C-terminal (C) domain. The MADS-box domain is considered to play an important role in controlling floral development in *Arabidopsis* and snapdragon (Weigel and Meyerowitz, 1994).

All B-function genes belong to the family of MADS-box genes and fall into either one of two different clades, namely *DEF*- or *GLO*-like genes (Theissen *et al.*, 1996, Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J. Mol. Evol. 43, 484-516). *DEF*- and *GLO*-like genes are closely related to the MADS-box gene family. These two clades together also represent a well supported gene clade (Theissen *et al.*, 1996). In addition to the higher eudicots, B genes have been most intensively studied in cereal grasses (family *Poaceae*), mainly the important crop plants and rice and maize model system (Moon *et al.*, 1999. Identification of a rice *APETALA3* homologue by yeast two-hybrid screening. Plant Mol. Biol. 40, 167-177; Ambrose *et al.*, 2000. Molecular and genetic analyses of the *silky1* gene reveal conservation in floral organ specification between eudicots and monocots. Mol. Cell 5, 569-579; Münster *et al.*, 2001. Characterization of three *GLOBOSA*-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. Gene 262, 1-13). In the literature, only one *DEF*-like gene has been reported in diverse monocots such as lily (*Lilium regale*), wheat (*Triticum aestium*), maize and rice (Münster *et al.*, 2001).

The orchid flower does not have the normal monocots and eudicots floral morphology. It has three sepals, three petals and one of the petals possesses a different morphological structure known as the lip. The male and female reproductive parts are combined in a uniform structure, the column, in the center of the flower. The pollen grains stick together to form pollinia located at the upper end of the column inside the anther (referring to FIG. 1a). As a reason, the result established in the model plant cannot be applied in constructing the mechanism of the elegant orchid floral morphology. In the prior art, the traditional breeding technique is still used in changing the orchid floral morphology. It spends a lot of time and the success rate is also low.

SUMMARY OF THE INVENTION

The invention provides four genes for controlling floral development in orchid after a study on the effect of the gene controlling floral development on the change of floral morphology in orchid. All of the four genes belonging to *DEF*-like genes have B-function of ABC model and the proteins encoded by them share a MIKC-type domain structure.

One subject of the invention is to provide an isolated nucleic acid molecule for controlling floral development in orchid, which nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule, PeMADS2, for controlling sepal development and the anti-sense strand thereof; (b) a nucleic acid molecule, PeMADS3, for controlling lip development and the anti-sense strand thereof; (c) a nucleic acid molecule, PeMADS4, for controlling lip and column developments and the anti-sense strand thereof; (d) a nucleic acid molecule, PeMADS5, for controlling petal and stamen developments and the anti-sense strand thereof; (e) one or more nucleic acid molecules hybridizing with the complement strand of any one of the nucleic acid molecules defined in (a), (b), (c) and (d) under stringent hybridization conditions; and (f) one or more nucleic acid molecules comprising the degeneration sequence of any one of the nucleotide

molecules defined in (a), (b), (c) and (d).

In another aspect, the invention provides a protein, a vector comprising the nucleic acid molecule, a cell, a protocorn, and a kit for controlling the floral development in orchid.

In still another aspect, the invention provides a method for producing transgenic orchid and a transgenic orchid produced according to the method, the method comprising the steps of:

(a) introducing the nucleic acid molecule according to the invention into an orchid cell to obtain an orchid transformed cell; and

(b) regenerating the orchid transformed cell to obtain the transgenic orchid plant.

In still another aspect, the invention provides a method for producing an orchid transformed cell comprising introducing the nucleic acid molecule according to the invention into an orchid cell to obtain the orchid transformed cell.

In still another aspect, the invention provides a method for controlling floral development in orchid, which comprises changing the expression of the protein according to the invention for controlling floral development.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1a illustrates the wild type flower of *Phalaenopsis equestris*. FIG. 1b illustrates the *P. equistris* peloric flower mutant. FIG. 1c illustrates the developmental stages of flower buds.

FIG. 2 illustrates alignment of the deduced amino acid sequences of PeMADS genes and some *GLO*- and *DEF*-like genes. The multiple alignment was generated by the computer program PILEUP and displayed by PRETTYBOX. Identity with consensus is denoted by black box.

Similarity with consensus is denoted by the gray, differences are indicated by white, gaps in the alignment are indicated by points, and positions that are not occupied by an amino acid by a '~'. The MADS-, I-, K-, and C-domains are indicated. OSMADS4 and OSMADS16 are from rice; SILKY1 is from maize; LMADS1 is from lily; DEF is from *Antirrhium*; PI and AP3 are from *Arabidopsis*; PeMADS2, PeMADS3, PeMADS4, and PeMADS5 are from *P. equestris*.

FIG. 3 illustrates alignment of the consensus sequences for PI-derived motif and paleoAP3 motif. PeMADS2, PeMADS3, PeMADS4, and PeMADS5 are from *P. equestris*; OSMADS16 is from rice; SILKY1 is from maize; TDR6 is from *L. esculentum*; TAMADS51 is from *T. aestivum*; SmAP3 is from *S. montevidensis*; PnAP3-2 is from *P. nudicaule*; MfAP3 is from *M. figo*; DeAP3 is from *D. eximia*; CMB2 is from *D. caryophyllus*; LMADS1 is from *L. longiflorum*.

FIG. 4 illustrates phylogenetic tree of B class MADS-box genes in the *DEF*, *GLO* and *GGM2* subfamilies. The phylogeny was conducted with the neighbor-joining algorithm. The tree was rooted using GMM13 which is member of sister clade of the B proteins as outgroup (Becker *et al.*, 2000. MADS-box gene diversity in seed plants 300 million years ago. Mol. Biol. Evol. 17, 1425-1434). The bootstrap values from 1000 replicates are indicated on most major nodes. Genus names of species, from which the respective genes were isolated, are given in the parentheses behind the protein names. Monocot proteins are highlighted by inverted boxes, and proteins from gymnosperms by open boxes. Proteins that are not boxed represent sequences from dicots. The monophyletic floral homeotic gene groups are marked by brackets at the right margin.

FIG. 5 illustrates Southern blot analysis of PeMADS genes in *P. equestris*. DNA gel blots containing 10 μg of genomic DNA digested with *Bgl* II (lanes 1, 4, 7, 10), *Eco* R I (lanes 2, 5, 8, 11), and *Hind* III (lanes 3, 6, 9, 12) were hybridized under stringent conditions with probes that were

derived from the 3'-specific region of *PeMADS* genes. The names of the respective genes are indicated at the top. The lengths of DNA markers (in kb) are indicated at the left margin.

FIG. 6 illustrates expression of *PeMADS* genes during the development of the flower buds and in different orchid tissues. The names of the respective genes are indicated at the right margin. Each lane contained 10 μg of total RNA from: stage I to stage IV flower buds (lanes 1 to 4), pedicles (lane 5), shoots (lane 6), leaves (lane 7), and roots (lane 8). Blots were hybridized with specific probes described in "Southern blot analysis". The 28S ribosomal RNA indicated the amount of total RNA loaded in each lane.

FIG. 7 illustrates Northern blot analysis of *PeMADS* genes in different floral organs of wild-type (FIG. 7a) and peloric mutant (FIG. 7b). RNA sources were sepals (Se) (lanes 1 and 6), petals (Pe) (lanes 2 and 7), lips (Li) (lanes 3 and 8), pollinium (Po) (lane 4), and Column (Co) (lanes 5 and 9) as indicated. The names of the respective genes are indicated at the right margin. The blot contained 10 μg of total RNA extracted from different mature floral organs in each lane. Blots were hybridized with specific probes described in "Southern blot analysis". The 28S ribosomal RNA indicated the amount of total RNA loaded in each lane.

DETAILED DESCRIPTION OF THE INVENTION

The present invention mainly relates to an isolated nucleic acid molecule for controlling floral development in orchid, which nucleic acid molecule is selected form the group consisting of:

(a) a nucleic acid molecule, *PeMADS2,* comprising the nucleotide sequence of SEQ ID NO: 1 and the anti-sense strand thereof;

(b) a nucleic acid molecule, *PeMADS3,* comprising the nucleotide sequence of SEQ ID NO: 3 and the anti-sense strand thereof;

(c) a nucleic acid molecule, *PeMADS4*, comprising the nucleotide sequence of SEQ ID NO: 5 and the anti-sense strand thereof;

(d) a nucleic acid molecule, *PeMADS5*, comprising the nucleotide sequence of SEQ ID NO: 7 and the anti-sense strand thereof;

(e) one or more nucleic acid molecules hybridizing with the complement strand of any one of the nucleic acid molecules as defined in (a), (b), (c) and (d) under stringent hybridization conditions; and

(f) one or more nucleic acid molecules comprising the degeneration sequences of any one of the nucleotide sequences of SEQ ID NO: 1, 3, 5, and 7.

According to the invention, the floral morphology of an orchid is changed if the amounts of the nucleic acid molecule and the gene product thereof are changed.

According to the invention, four expressed sequence tags (ESTs) of *DEF*-like genes are obtained from the cDNA clones of *Phalaenopsis equestris* flower buds. Four *DEF*-like ESTs were identified in the assembled EST database and the full-length sequences of these genes were cloned and characterized. The results showed that the *P. equestris* genome contains at least four different *DEF*-like genes, namely *PeMADS2*, *PeMADS3*, *PeMADS4* and *PeMADS5*. The gene *PeMADS2* encodes a 227-amino acid PeMADS2 protein having a sequence given in SEQ ID NO: 2. The gene *PeMADS3* encodes a 222-amino acid PeMADS3 protein having a sequence given in SEQ ID NO: 4. The gene *PeMADS4* encodes a 224-amino acid PeMADS4 protein having a sequence given in SEQ ID NO: 6. The gene *PeMADS5* encodes a 219-amino acid PeMADS5 protein having a sequence given in SEQ ID NO: 8.

Referring to FIG. 2, the PeMADS2, PeMADS3, PeMADS4, and PeMADS5 proteins share a typical MIKC-type domain structure in multiple

sequence alignments with other MADS-box proteins. The PeMADS proteins of the invention include:

(a) an MADS domain, at the amino acids 1 to 57 in the PeMADS proteins, which is highly conserved (86-96%);

(b) an intervening domain, at the amino acids 58 to 76 in the PeMADS proteins;

(c) a domain AP3 that is a highly conserved, at the amino acids 83 to 87, having a sequence (H/Q)YEXM in PeMADS2, PeMADS3 and PeMADS4, but having a sequence QYQRM in PeMADS5;

(d) a keratin-like domain, at the amino acids 77 to 151 of the PeMADS proteins;

(e) a C-terminal domain, at the amino acids 152 to the last one of PeMADS proteins.

As shown in FIG. 3, there is a PI-derived motif (FXFRLOPSQPNLH) in PeMADS2, PeMADS3 and PeMADS4, which is of more than 60 % homology and a paleoAP3 motif (YGXHDLRLA). (Moon, Y.-H. *et al.*, 1999. Identification of a rice *APETALA3* homologue by yeast two-hybrid screening. Plant Mol. Biol. 40, 167-177.) However, PeMADS5 shows no significant similarity. Except the Pi-derived motif and paleoAP3 motif, the C-terminal domains of the PeMADS genes have a great variation, which suggests that the four PeMADS genes identified in the EST database indeed exist.

The study in topology of the phylogenetic trees having the PeMADS2, PeMADS3, PeMADS4, PeMADS5 and other MADS-box genes shows that the four genes according to the invention belong to the clade of *DEF*-like genes, wherein *PeMADS2/PeMADS5* and *PeMADS3/PeMADS4* share two common ancestors, from which they were derived by gene duplication (as shown in FIG. 4). Furthermore, a study by

using a C-terminal domain as a probe shows that *PeMADS2*, *PeMADS4*, and *PeMADS5* in *P. equestris* have a single copy of genes, respectively, and *PeMADS3* has two copies (as shown in FIG. 5).

In the floral development in orchid, four stages according to bud length are usually defined as: stage I: 0-1 mm; stage II: 1-2 mm; stage III: 2-5 mm; stage IV: 5-10 mm. The expressions of the different *PeMADS* genes in the buds vary at the different stages: *PeMADS2*, *PeMADS3* and *PeMADS4* genes are expressed at stages II and III, and *PeMADS5* gene is expressed at stage IV (as shown in FIG. 6). Such genes are expressed in the buds from the early stage to the end of the floral development. However, the *PeMADS* genes are not expressed in other organs such as pedicle, shoot, leaf, or root.

The expressions of the *PeMADS* genes vary in different floral organs in the wild-type orchid: *PeMADS2* is expressed strongly in sepal, petal and less in column, but not in lip and pollinium; *PeMADS3* is expressed strongly in petal, lip and less in column, but not in sepal and pollinium; the expression of *PeMADS4* is only detected in lip and column; and *PeMADS5* is expressed predominantly in sepal, petal, and lip and weakly in column (referring to FIG. 7a).

The expressions of *PeMADS* genes of the wild-type orchid are compared with those of the mutant orchid. The mutant (referring to FIG. 1b) comprises three sepals, three lip-like petals, an arching column, a nearly reduced anther, and a flat stigma, but have no stamen primordial and pollinium. In the mutant flower, the expressions of the *PeMADS* genes are listed below: *PeMADS2* is expressed in sepal, lip-like petal, column, and weakly expressed in lip; *PeMADS3* is strongly expressed in lip-like petal, lip and column. Both of the above-mentioned genes are more strongly expressed in column of the mutant flowers than those of the wild type. *PeMADS4* is strongly expressed in lip and column and weakly expressed in lip-like petal. Most strikingly, the expression of *PeMADS5* was completely

abolished in all floral organs of the mutant flower. These results suggest that both the positive regulation expression of *PeMADS4* and the negative regulation of *PeMADS5* play important roles in both petal and lip formation during the orchid floral development.

In the wild-type orchid according to the invention, *PeMADS2* and *PeMADS5* are strongly expressed in sepal and petal while *PeMADS3* and *PeMADS4* are not expressed in sepal. On the other hand, in the mutant orchid, only *PeMADS2* is expressed in the sepal. In view of both the wild-type and mutant orchids both having sepal, *PeMADS2* and the gene product thereof, PeMADS2, regulate sepal development. Controlling the sepal development is achieved by changing the amounts of *PeMADS2* and the gene product thereof, PeMADS2, in an orchid or in an orchid cell.

In the wild-type orchid according to the invention, *PeMADS3* and *PeMADS4* are strongly expressed in lip while *PeMADS2* and *PeMADS5* are weakly expressed. On the other hand, in the mutant orchid, *PeMADS4* is strongly expressed in the lip-like petal. *PeMADS3* and *PeMADS4* and the gene products thereof, PeMADS3 and PeMADS4, regulate lip development, and *PeMADS4* and the gene product thereof, PeMADS4, possesses a different morphological structure, which is known as the lip. Controlling the lip development is achieved by changing the amounts of *PeMADS4* and the gene product thereof, PeMADS4, in an orchid or in orchid cells.

In the wild-type orchid according to the invention, all *PeMADS* genes are not expressed in pollinium while *PeMADS4* is expressed more strongly than others. It shows that *PeMADS4* and the gene product thereof, PeMADS4, regulate column development. Controlling the column development is achieved by changing the amounts of *PeMADS4* and the gene product thereof, PeMADS4, in orchid or in orchid cells. Furthermore, as shown in the comparison between the expression of *PeMADS* genes in wild-type and mutant column, *PeMADS2* and *PeMADS3* are expressed more strongly in the wild-type column than those in the mutant, while the

expression of *PeMADS5* is not detected in column. In view of the mutant lacking its stamen primordial, *PeMADS5* and the gene product thereof, PeMADS5, regulate stamen primordial development. Controlling the stamen primordial development is achieved by changing the amounts of *PeMADS5* and the gene product thereof, PeMADS5, in an orchid or in orchid cells.

In the wild-type orchid according to the invention, all *PeMADS* genes except *PeMADS4* are expressed in petal. That means that *PeMADS4* does not contribute in petal development. On the other hand, in the mutant orchid, *PeMADS5* is not expressed in the petal. It is concluded that *PeMADS5* and the gene product thereof, PeMADS5, regulate petal development. Controlling the petal development is achieved by changing the amounts of *PeMADS5* and the gene product thereof, PeMADS5, in an orchid or in orchid cells.

As used herein, the term "anti-sense strand" refers to a nucleic acid molecule able to hybrid to the RNA transcripts of *PeMADS* genes under appropriate conditions. In the preferred embodiment of the invention, the anti-sense strand is a nucleic acid molecule that is complement to the RNA transcripts of the *PeMADS* genes. The appropriate conditions for such hybridization are the physiological or equivalent conditions found within plant cells including those found in the nucleus and cytoplasm or the standard *in vitro* conditions normally used by skilled persons in the art to determine sequence homology between two nucleic acids, such as at the *in vitro* conditions ranging from moderate (about 5 X SSC at 52 °C) to high (about 0.1 X SSC at 65 °C.) stringency conditions.

As used herein, the term "degeneration sequence" refers to a nucleotide sequence that encodes the gene products of *PeMADS* genes, i.e. PeMADS proteins besides *PeMADS* genes.

The present invention also provides a vector for controlling floral development in orchid comprising the nucleic acid molecule according to

the invention. The vector is used for storing or producing the nucleic acid molecule, or introducing the nucleic acid molecule into a plant or a plant cell. Preferably, the vector is a shuttle vector. As used herein, the term "shuttle vector" refers to a vector, which can be manipulated and selected in both a plant and a convenient cloning host, such as a prokaryote. Such a shuttle vector may include a kanamycin resistance gene for selection in plant cells and an actinomycin resistance gene for selection in a bacterial host. Besides, the shuttle vector contains an origin of replication appropriate for the prokaryotic host used, and preferably at least one unique restriction site or a polylinker containing unique restriction sites to facilitate the construction.

In another aspect, the nucleic acid molecule according to the invention is preferably driven by a promoter. More preferably, the promoter has an ability to drive expression of a nucleic acid within at least one portion of the reproductive tissues in the recipient plant, such as the cauliflower mosaic virus 35S protein promoter, the α-1 and β-1 tubulin promoter, and the histone promoters. In one embodiment of the invention, the promoter is an inducible promoter comprising but not limited to heat-shock protein promoters and light-inducible promoters including the three chlorophyll a/b light harvesting protein promoters. The methods of vector construction are well known to those skilled in the art.

The present invention also provides a kit comprising the vector according to the invention. The kit is used for controlling floral development in orchid. For convenient operation, the kit further comprises a buffer solution needed in the transformation process.

In another aspect, the invention provides a transformed cell with the vector comprising the nucleic acid molecule for controlling floral development in orchid according to the invention. The cell may be a prokaryote cell or an orchid cell, and preferably, a *Phalaenopsis spp.* cell. As used herein, the term "transformation" refers to a process for changing

the genetic material of a cell through introducing a nucleic acid molecule. Persons skilled in this art can conduct the transformation according to the disclosure of the invention and normal knowledge in molecular biology. For example, the vector may be introduced into a bacterial by heat shock process, or the vector is introduced into a plant cell by a gene gun. The invention also provides a transgenic orchid comprising at least one cell transformed with the vector, which may be preformed by conventional methods known to persons skilled in the art.

The invention also provides a method for producing an orchid transformed cell comprising introducing the nucleic acid molecule into the orchid cell to obtain the orchid transformed cell.

In another aspect, the invention provides a method for producing a transgenic orchid comprising the steps of:

(a) introducing the nucleic acid molecule according to the invention into an orchid cell to obtain an orchid transformed cell; and

(b) regenerating the orchid transformed cell to obtain the transgenic orchid plant.

In the embodiment of the invention, a transgenic orchid plant may be produced through a protocorn-like body in vegetative planting or aspetic seed germination. The term "protocorn-like body" used herein refers to a tissue, which has a potential to differentiate and is an ability for strong and rapid proliferation ability. After separating the cells in a protocorn-like body, each can regenerate a new protocorn-like body and then a new plant. In step (a), the nucleic acid molecule is introduced into a protocorn-like body, and preferably through a gene gun. At this moment, the nucleic acid molecule is introduced into some cells in the protocorn-like body to form transformed cells, and some cells are not introduced with the molecule. The transformed cells can be selected with the marker of the vector. In step (b), the transformed cells are regenerated to transgenic plants. As used

herein, the term "regeneration" refers to a growth process of a plant from a plant cell, a group of plant cells or a part of a plant. The method of regeneration is well known to persons skilled in this field. A transgenic orchid produced thereby is also provided in the invention.

In still another aspect, the invention provides a protein for controlling floral development in orchid, and preferably, *Phalaenopsis spp.* The protein is encoded by the nucleic acid molecule according to the invention. In a preferred embodiment of the invention, the protein is selected from the group consisting of PeMADS2, PeMADS3, PeMADS4, and PeMADS5, and wherein the PeMADS2, for controlling sepal development, has the amino acid sequence as given in SEQ ID NO: 2; the PeMADS3, for controlling lip development, has the amino acid sequence as given in SEQ ID NO: 4; the PeMADS4, for controlling lip and column developments, has the amino acid sequence as given in SEQ ID NO: 6; the PeMADS5, for controlling petal and stamen developments , has the amino acid sequence as given in SEQ ID NO: 8.

The invention also provides a method for controlling floral development in orchid, which comprises changing the expression of the proteins for controlling floral development in orchid. According to the invention, the method of changing the expression of the proteins comprises the steps of inducing, inhibiting and deleting the expression. In one embodiment of the invention, the expression of the proteins can be changed by increasing or decreasing the ploid of the nucleic acid molecule encoding the proteins in at least one cell of the plant. In a preferred embodiment of the invention, a gene gun is used to introduce the nucleic acid molecule into the cell for changing the expression of the protein. In another embodiment of the invention, the expression of the proteins can be changed by introducing an anti-sense strand of the nucleic acid molecule into the cell. In one embodiment of the invention, the cell is derived from a protocorn-like body.

As used herein, the phrase "changing floral morphology" refers to a physical modification in the structure of a plant's reproductive tissue as compared to the parent plant. In an embodiment of the invention, a transgenic plant can be obtained by regenerating a transformed plant cell with the genes of the invention that are capable of modifying the phenotype of the plant, wherein the cells of the transgenic plant all have the same genetic material. In another embodiment of the invention, a mosaic plant can be obtained by transforming some of cells in a plant, such as reproductive cells or tissues, with the genes of the invention, wherein only the transformed cells express the modified phenotype as compared to the parent plant.

According to the invention, the plants to be transformed with the genes include orchid and orchid cells, preferably *Phalaenopsis spp.*, which may be the wild type and an artificial mutant that produced by such as chemical modification, X-ray activated random mutagenesis or recombinant techniques.

The following Examples are given for the purpose of illustration only and are not intended to limit the scope of the present invention.

Example:

Plant materials and RNA preparation: The wild type *P. equestris* having red sepals and orange petals (referring to FIG. 1a) and its peloric mutant having lip-like petals, an arching column, a nearly reduced anther, a flat stigma and no stamen primordial and pollinium (referring to FIG. 1b) were grown in the greenhouses at Taiwan Sugar Research Institute (TSRI) with natural light and controlled temperatures ranged from 23°C to 27°C.

For RNA extraction, roots, leaves, shoots, developing flower buds from stages I-IV (referring to FIG. 1c), and various organs of flower buds including sepals, petals, lips, pollinium, and columns of developing flowers (stage IV) were collected, immersed in liquid nitrogen, and stored at -80°C

until used for RNA extraction. Total RNA was extracted following the method described by O'Neill *et al.* (O'Neill, S. D. *et al.* 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell 5, 419-32.)

cDNA library construction: Poly $(A)^+$ mRNA was prepared from stage IV flower buds of *P. equestris* using Poly (A) Quick RNA Isolation kit (Stratagene®, La Jolla, CA). The synthesis of cDNA, size selection, addition of linkers, insertional ligation, and packaging into λ vector were carried out following the manufacture's instructions (λZAPII, Strategene®). The total primary titer of cDNA library was 1.1×10^6 pfu/ml. The phage library was converted to the plasmid form by mass excision according to the manufacture's protocol (Strategene®). The resulting phagimid library was plated at low density on Luria Bertani agar (LBA) plates containing kanamycin (25 mg/L). Over 4,000 randomly selected bacterial colonies from cDNA library were cultured for plasmid isolation, nucleotide sequencing and long-term storage in microtiter plates.

Plasmid DNA was purified from *E. coli* cultures by alkaline lysis following standard protocol, vacuum filtration, and anion-exchange chromatography using high-throughput, 96-well format system (Qiagen®, Ontario, Canada). The sequencing processes were performed by using the standard T3 sequencing primer. The sequence to the presumed 5' end of each cDNA was determined. Automated cycle sequencing of DNA was carried out using dye-labeled terminators, and the products were resolved by electrophoresis through acrylamide gels (ABI 377, Applied Biosystems ®, Foster City, CA).

Sequence data analysis: Raw DNA sequence data were edited to remove vector sequences, polyA sequences and poor quality data using a computer program (Sequencher 4.1, GeneCode®, Ann Arbor, MI). Computer-processed sequences were checked manually, compared with electropherograms, and further edited, if necessary to improve the quality

and reliability of the data. Each edited EST was translated in all six reading frames and compared with the non-redundant database at the National Center of Biotechnology Information (NCBI) using the BLASTX program (Altschul *et al.*, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402). Default BLAST parameter values were used. The sequences that had no significant similarity were further compared by BLASTN (Altschul *et al.*, 1997).

5' rapid amplification of PeMADS cDNA ends: Rapid amplification of cDNA ends (RACE) was carried out to obtain the full-length cDNA by extending the 5' ends of cDNA using the SMART RACE cDNA amplification kit (Clontech®, Palo Alto, CA). In the amplification, the first strand of the cDNAs for 5' RACE was obtained from 400 ng total RNA of stage IV flower buds, the cDNA containing the 5' end for *PeMADS* clones were obtained by the PCR amplification using 5'-specific universal primer (Clontech®) and 3' gene-specific primer for different *PeMADS* genes. The gene specific primers for *PeMADS2*, *PeMADS3*, *PeMADS4*, and *PeMADS5* are 5'-TCT CTC TGA ATA GAT CC C CCA TCT C-3' (SEQ ID NO: 9), 5'-GCA GTG CTA GAC CCT ACT TGT AAG C-3' (SEQ ID NO: 10), 5'-GCT ATA TCC CGT TCC TTG AAG ATT TTG-3' (SEQ ID NO: 11), and 5'-TCC TAT GAT GTT AAG CCA TGA AAA C-3' (SEQ ID NO: 12), respectively. The thermal cycling protocol consisted of an initial denaturation at 94°C for 5 sec., followed by 25 cycles at 94°C for 30 sec., 65°C for 30 sec. and 72°C for 2 min. and a final extension at 72°C for 5 min. RACE-products were re-amplified with the *PeMADS* nested gene-specific primers and the nested universal primer provided in the RACE kit. The nested gene-specific primers for *PeMADS2*, *PeMADS3*, *PeMADS4*, and *PeMADS5* are 5'-TGA TTC GGA TGA ACA ACC CTA-3' (SEQ ID NO: 13), 5'-AGG AAG CCC CAT TTC CAA GTG-3' (SEQ ID NO: 14), 5'-GTG CAT TAA GTT CCG GTG TGT-3' (SEQ ID NO: 15), and 5'-TGC ACA TTT GGC TCA CTC CGG-3' (SEQ ID NO: 16), respectively.

The PCR protocol consisted of an initial denaturation at 94°C for 5 min. followed by 30 cycles at 94°C for 30 sec., 60°C for 2 min., 72°C for 2 min. and a final extension at 72°C for 5 min. The PCR products were cloned into pGEM-T Easy vector (Promega®, Madison, WI) and sequenced on both strands from ten positive clones.

Sequence alignments and construction of phylogenetic trees: Pairwise alignments of conceptual amino acid sequences were generated using the GAP program of the GCG package (Wisconsin Package Version 10.3, Accelrys Inc®., San Diego, CA) with a gap weight of 8 and a gap length weight of 2 (default parameters). Multiple alignments were generated using the PILEUP program (Wisconsin Package Version 10.3) of the same package with the same alignment parameters. The results are shown in FIGs. 2 and 3. Table 1 provides the percentage of amino acid identity and similarity at MADS-domain and the full-length sequence of DEF-like proteins from *Arabidopsis*, rice, maize, lily, and orchid.

Table 1:

	AP3	OSMADS16	SILKY1	LMADS1	PeMADS2	PeMADS3	PeMADS4	PeMADS5
AP3	—	75.0[a]/48.9[b]	76.7/51.1	73.3/51.8	75.0/48.2	71.7/48.6	73.3/49.1	70.0/47.7
OSMADS16	80.0[c]/55.7[d]	—	98.3/90.5	81.7/69.8	80.0/60.8	80.0/65.2	81.7/63.5	76.7/54.4
SILKY1	81.7/58.0	98.3/93.7	—	93.3/69.6	81.7/60.3	81.7/66.7	83.3/65.0	78.3/51.1
LMADS1	83.3/61.4	88.3/76.6	90.0/75.4	—	85.0/66.2	85.0/78.0	86.7/76.3	80.0/58.3
PeMADS2	83.3/	90.0/71.2	91.7/71.4	93.3/78.2	—	86.7/64.0	86.7/67.0	86.7/68.3

	60.5							
PeMADS3	80.0/ 57.8	88.3/73.3	90.0/73. 9	88.3/85.6	91.7/75.2	—	91.7/80.6	83.3/60.3
PeMADS4	81.7/ 60.5	91.7/73.9	93.3/74. 0	91.7/86.2	95.0/78.1	96.7/87.4	—	80.0/58.3
PeMADS5	80.0/ 61.2	81.7/63.7	83.3/60. 3	85.0/69.3	90.0/75.7	88.3/71.5	86.7/69.4	—

[a]: percentage of amino acid identity at MADS-domain of DEF-like proteins

[b]: percentage of amino acid identity of the full-length sequence of DEF-like proteins

[c]: percentage of amino acid similarity at MADS-domain of DEF-like proteins

[d]: percentage of amino acid similarity of the full-length sequence of DEF-like proteins

The result evidenced that all PeMADS proteins have specific MIKC-type domain, and *PeMADS* genes are MADS of *DEF*-like genes.

The sequences used for phylogenetic analysis includes the MADS-box domain plus the 110 amino acid downstream from the MADS-box domain (Purugganan *et al.*, 1995. Molecular evolution of flower development: diversification of the plant MADS-box regulatory gene family. Genetics 140, 345-356). Phylogenetic trees were constructed by the neighbor-joining method and evaluated by bootstrap analysis as described (Münster *et al.*, 1997). The result is shown in FIG. 4. It demonstrates that all the four proteins belong to B class genes from monocots, and wherein *PeMADS2/PeMADS5* and *PeMADS3/PeMADS4* share a common ancestor from which they were derived by gene

duplication.

Isolation of genomic DNA and Southern blot analysis: Genomic DNAs were isolated from leaves following the method described by Carlson *et al.* (1991) (Carlson, L. E. *et al.* 1991. Segregation of random amplified DNA markers in F1 progeny of conifers. Theor. Appl. Genet. 83, 194-200). Genomic DNA samples were digested with restriction enzymes *Bgl* II, *Eco* R I and *Hind* III, resolved in 0.8 % agarose gels, and transferred to nylon filters (Amersham Pharmacia Biotech®, Piscataway, NJ) using a vacuum transfer system (Amersham Pharmacia Biotech®). The conserved MADS domain and most of K domain were excluded in the probes to avoid cross hybridization. The *PeMADS2*-specific probe (295 bp) was generated by PCR with *PeMADS2*-specific internal primer pair 5'-GAA ACT TAC CGC GCT CTA-3' (SEQ ID NO: 17) and 5'-TCT CTC TGA ATA GAT CCC CCA TCT C -3' (SEQ ID NO: 18). The PeMADS3-specific probe (284 bp) was amplified with *PeMADS3*-specific internal primer pair 5'-CTC TCA AGA AAC CCA CAG-3' (SEQ ID NO: 19) and 5'-GCA GTG CTA GAC CCT ACT TGT AAG C-3' (SEQ ID NO: 20). The *PeMADS4*-specific probe (296 bp) was amplified by using *PeMADS4*-specific internal primer pair 5'-GAG GAC CAC CCA GTG TAT-3' (SEQ ID NO: 21) and 5'-CAC AGA ATC ACA CAT AGC A-3' (SEQ ID NO: 22). The *PeMADS5*-specific probe (289 bp) was amplified by using *PeMADS5*-specific internal primer pair 5'-CAA ACA GAC ACT TGC AGG-3' (SEQ ID NO: 23) and 5'-TCC TAT GAT GTT AAG CCA TGA AAA C-3' (SEQ ID NO: 24). In Southern blots, the ^{32}P-labeled probes was used for hybridization, and the pre-hybridization and hybridization were performed following the standard protocols. The result is shown in FIG. 5. It is evidenced that *PeMADS2*, *PeMADS4* and *PeMADS5* have a single copy and *PeMADS3* has two copies.

RNA blot analysis: For Northern blot hybridization, Ranks were prepared from various organs of *P. equestris* plants, including stages I to IV flower buds, pedicles, shoots, leaves and roots. Total RNA samples from

various floral organs, including sepal, petal, lip, pollinium and column, were prepared. Ten μg of total RNA samples were denatured with glyoxalin, subjected to electrophoresis on a 1% agarose gel, and transferred to nylon filters (Amersham Pharmacia Biotech®). The RNA blots were hybridized with the same probes as described above. The pre-hybridization and hybridization were performed following the standard protocols. For internal control, a partial fragment of 45S rDNA was used as the probe (a gift from Dr. Y. Y. Kao, Department of Botany, National Taiwan University), and the 28S rRNA band was detected.

The results of the *PeMADS* gene expression in different stages of flower bud development and in different orchid tissues are shown in FIG. 6. *PeMADS2*, *PeMADS3* and *PeMADS4* expressed in stage II and III, and *PeMADS5* expressed in stage IV. It is evidenced that these genes express from early stage to the end of flower bud development. On the other hand, these *PeMADS* genes did not express in other plant tissues such as pedicles, shoots, leaves, and roots. The results of Northern blot of PeMADS genes in different tissues of the wild type and mutant are shown in FIGs. 7a and 7b. In the wild type, *PeMADS2* was expressed strongly in sepal, petal and less extent in column, but not in lip and pollinium; *PeMADS3* was expressed strongly in petal, lip and less in column, but not in sepal and pollinium; the expression of *PeMADS4* was detected in lip and column only; and *PeMADS5* was expressed predominantly in sepal, petal, and lip and weakly expressed in column. In the mutant, *PeMADS2* was expressed in sepal, lip-like petal, column, and weakly in lip; *PeMADS3* was expressed strongly in lip-like petal, lip and column. Both of the genes had stronger expressions in column of the mutant flowers than the wild type. *PeMADS4* was expressed strongly in lip and column and weakly in lip-like petal, and the expression of *PeMADS5* was completely abolished in all floral organs of the mutant flower.

While embodiments of the present invention have been illustrated and described, various modifications and improvements can be made by

persons skilled in the art. The present invention is not limited to the particular forms as illustrated, and that all the modifications not departing from the spirit and scope of the present invention are within the scope as defined in the appended claims.